RNA (tube based) QC SOP (For Collaborators)

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Summary

Before shipping your RNA sample(s), please be sure to follow the JGI sample preparation and sample submission guidelines located at http://my.jgi.doe.gov/general/gettingstarted.html

This protocol describes how to perform quality control of RNA samples to evaluate the quantity (Qubit RNA BR Assay) and quality (Agilent Bioanalyzer, RNA 6000 Nano Kit) of your RNA sample(s). We recommend all RNA samples to be evaluated with this protocol before they are shipped to JGI.

Materials & Reagents

Materials/Reagents/Equipment	**Supplier/Vendor**	**Stock Number**
Disposables		
Qubit assay tubes		Q32856
10 ml Serological pipettes		1367811E
0.2 ml PCR 8-tube flex-free strip (RNase-free)		1402-4700
0.2 ml PCR tubes (individual)	Ambion/Life Technologies	AM12225
Bioanalyzer Cleaning Chips	Agilent	5065-9951
Petri Dishes, 100x15 mm, BD Falcon		25373-100
1.5 ml microcentrifuge tubes (RNase-free)	Ambion	AM12450
Air canister		23022523
Agilent RNA 6000 Nano Kit and Chips (includes syringe for Priming Station)	Agilent	5067-1511
Reagents		
RNase Zap	Ambion	AM9780
DNA AWAY Surface Decontaminant	Fisher Scientific	21-236-28
70% Isopropanol	Fisher Scientific	19-130-3860
Nuclease Free Water (10 × 50 ml)	Ambion	AM9937
Qubit RNA BR Assay Kit	Invitrogen	Q10211
Equipment		
96-well cold block	E&K Scientific	EK-76120

Bioanalyzer Chip Priming Station	Agilent	5065-4401
Chip Vortexer	IKA	3617036
2100 Electrophoresis Bioanalyzer	Agilent	G2939AA
Qubit 2.0 Fluorometer	Invitrogen	Q32866
Thermocycler	Applied Biosystems	N8050001
Microcentrifuge	Eppendorf	022620100
Serological pipettor	Eppendorf	022230204
Speed Vac	Thermo Fisher	SPD1010

EH&S

PPE Requirements:

Safety glasses, lab coat, and nitrile gloves should be worn at all times while performing this protocol. Additional safety equipment is required at designated steps

Procedure

1. Collect RNA Samples for QC.
   1.1 Until ready to begin lab work, store RNA in a -80^{0}C freezer.
2. Prepare lab bench for use with RNA samples.
   2.1 Clean pipettes with RNase Zap and kimwipes. Wipe lab bench and tube racks with RNase Zap. Repeat cleaning pipettes, lab bench, and tube racks with DNA away, and then with 70% Isopropanol.
3. Measure and record the volume of each RNA sample.
   3.1 After removing RNA samples from the -80^{0}C freezer, immediately transfer to ice. Thaw all RNA samples on ice. Briefly centrifuge tubes to collect droplets from tube wall and lid.
   3.2 Pipette one RNA sample at a time until the exact volume is determined. Record the volumes.
4. **Measure the quantity** of RNA samples using the Qubit RNA BR Assay Kit.
   4.1 Review the Qubit RNA BR Assay Kit manual to become familiar with the protocol before beginning the assay (https://tools.invitrogen.com/content/sfs/manuals/mp10210.pdf). The Qubit RNA reagent and buffer should be stored at room temperature, and the Qubit RNA reagent should be stored in the dark. The 2 RNA standards should be stored at 4^{0}C.
   4.2 Vortex RNA samples ~ 4 seconds, centrifuge briefly, and dilute an aliquot as needed based on reported collaborator concentrations (e.g., if reported concentration is 4000 ng/µl, dilute 1:16 so that the sample will be within Qubit and Nano Chip quantitative range). Dilutions can be made in strip tubes, and stored on a 96 well cold block.
   4.3 Set up the required number of 0.5 ml Qubit assay tubes for standards and samples. Label tube lids.
   4.4 Make sufficient Qubit working solution for the total number of reactions (standards and samples), by combining 1 µl RNA BR Reagent to 199 µl RNA BR Buffer for each reaction:

# reactions	1	30
RNA BR **Buffer**	199	5970
RNA BR **Reagent**	1	30
total (µl)	200	6000

4.5 Add 190 µl Qubit working solution to the 0.5 ml tubes for standards. Add 10 µl of each Qubit RNA BR standard to the appropriate tube, and mix by vortexing ~ 4 seconds. If there are bubbles, briefly centrifuge.

4.6 Add 198 µl Qubit working solution to the 0.5 ml tubes for samples. Add 2 µl RNA sample (diluted if necessary), and mix by vortexing ~ 4 seconds. If there are bubbles, briefly centrifuge. Incubate all tubes at room temperature for 2 minutes.

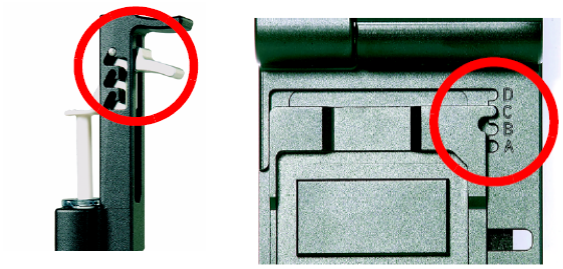
4.7 On the Qubit 2.0 Fluorometer, press RNA, then RNA Broad Range, press Yes to run a new calibration, then insert the tube containing Standard #1 into the Fluorometer. Close the lid, and press read. Insert the tube containing Standard #2, close the lid, and press read. Insert the tube containing RNA sample, close the lid, and press read. Record the concentration. Repeat for all RNA samples.

4.8 Multiply concentration values by 100, and by the RNA dilution factor (if RNA samples were diluted to be within the quantitative range). Convert the concentration units to ng/µl.

5. **Test RNA quality** by using the Agilent Bioanalyzer, RNA 6000 Nano Kit.

5.1 Dilute RNA samples if needed based on Qubit concentrations to be within the RNA 6000 Nano Kit quantitative range (5 to 500 ng/µl). If dilutions were used for quantifying RNA samples by Qubit, and the dilutions are also within the Nano Chip range, the same diluted RNA sample can be used.

5.2 Review the Agilent RNA 6000 Nano Kit Guide to become familiar with the protocol before beginning the assay (http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90035_QuickRNA6000Nano.pdf). The RNA ladder should be stored in Ambion 0.2 ml individual PCR tubes, as aliquots of 2 µl at -80^{0}C, and the Nano Kit should be stored at +4^{0}C. Before beginning the assay, allow the Nano Kit to be at room temperature for at least 30 minutes. Ensure that the chip priming station is only used for RNA chips. Replace the syringe every 1-2 weeks on the chip priming station. The clip on the priming station should be on the highest of the 3 levels, and the base plate should be at position C:



5.3 Decontaminate the Bioanalzyer electrodes.

a. Prepare three electrode cleaner chips as follows:

i. Prepare 0.5X solution of RNase Zap (e.g., 20 ml RNase Zap, 20 ml nuclease-free water). Label one of the electrode cleaner chips "RNase Zap", and fill chip with 350 µl of 0.5X RNase Zap.
ii. Label the other two chips "H2O #1" and "H2O #2". Fill each with 450 µl nuclease-free water. Ensure the liquid in all three chips is evenly distributed with no bubbles.
iii. To protect the cleaner chips from contamination while not in use, keep each one in a BD Falcon petri dish.

b. Insert the RNase Zap electrode cleaner chip into the Bioanalyzer, close the lid, and wait 60 seconds. Open the lid, insert the H2O #1 chip, leave for at least 2 minutes, and repeat with the H2O #2 chip. During these steps, you can proceed with the next steps (prepare gel-dye mix, gather the RNA ladder aliquot, and pre-heat the thermocycler).

c. Dry the electrodes using an air canister for ~ 10 seconds.

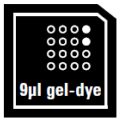
5.4 Pipette 550 µl RNA Nano gel matrix into spin filter. Centrifuge for 10 minutes at room temperature in Eppendorf microcentrifuge at > 10,000 rpm. Aliquot 65 µl filtered gel into 1.5 ml tube. Vortex RNA Nano dye concentrate briefly, and add 1 µl into 65 µl filtered gel tube. Vortex solution well, and centrifuge for 10 minutes at room temperature in Eppendorf microcentrifuge at > 10,000 rpm. Make gel-dye mix fresh for each use.

5.5 Pre-heat the thermocycler to 70^{0}C. Remove 1 RNA Nano Ladder aliquot from -80^{0}C freezer, and place on cold block.

5.6 Denature RNA samples and RNA Nano Ladder on thermocycler for 2 minutes at 70^{0}C. Transfer to cold block after denaturation.

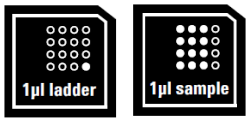
5.7 Place a new RNA Nano chip on the chip priming station. Pipette 9 µl of gel-dye mix into the following well:

5.8 With the plunger at the 1 ml position, close the chip priming station. Press the plunger until it is held by the clip. Wait 30 seconds, then release clip. Wait 5 seconds and slowly pull plunger back to 1 ml position. Open the chip priming station and pipette 9 µl of gel-dye mix into the following wells:



5.9 Pipette 5 µl of RNA Nano marker in all 12 sample wells and the ladder well:

5.10 Pipette 1 µl of prepared ladder and 1 µl of prepared RNA sample in the following wells:



5.11 Place the chip in the IKA vortexer and vortex 1 minute at 2100 rpm (2400 rpm is in the Agilent protocol, however may splatter samples). Place the chip in the Bioanalyzer instrument within 5 minutes of preparation. Run the Nano Chip using either the Eukaryote Total RNA Nano or Prokaryote Total RNA Nano software setting, depending on the RNA sample type.

5.12 After the Nano Chip run completes on the Bioanalyzer, clean electrodes as before, and dry with air canister. Electrode cleaner chips can be reused for up to one week (store them upside down in petri dishes after the cleaning solution has been removed). Record the following data from the run: concentration in ng/µl and RIN value. Analyze RNA quality using the electropherograms. Label rRNA peaks or lower marker if necessary.

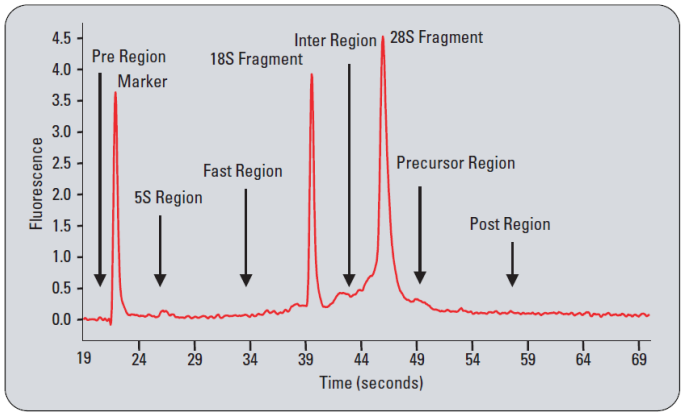


Figure 3
Electropherogram detailing the regions that are indicative of RNA quality.

5.13 Return RNA samples to the -80^{0}C freezer.

5.14 For all non-Metatranscriptome RNA samples use the following specifications to determine if RNA samples pass or fail:

a. Fail if total RNA mass is insufficient for required aliquot size.
   i. 2µg ±10% aliquot mass for standard RNA-Seq with rRNA Depletion library construction.
   ii. 50ng ±10% aliquot mass for RNA-Seq without rRNA Depletion library construction.
   iii. 5µg ±10% aliquot mass for Small RNA library construction.
   iv. 10ng ±10% aliquot mass for Low Input RNA-Seq library construction.

b. There should be 2 distinct rRNA peaks, with the large ribosomal subunit higher than the small subunit.
   i. Fail the sample if the large ribosomal subunit is significantly smaller than the small subunit (e.g., the large ribosomal subunit is half the height of the small ribosomal subunit).

ii. Ideally a sample with no degradation has an rRNA ratio close to 2.0; however with the exception of RNA prepared from cultured cells, it is rare to see an rRNA ratio of 2.0 or greater. There is some debate over what can potentially contribute to variability in rRNA ratios (tissue type, high degree of secondary and tertiary structure in the large ribosomal subunit which may easily re-nature after the denaturation at 70C for 2 minutes, concentration of the RNA sample, etc.). If the large ribosomal subunit is smaller than the small subunit, but not so much that the sample would fail just for that, then consider if there is visible degradation in the sample (e.g., elevated baseline, or large peak in the 5S region). A poor rRNA ratio coupled with visible degradation is a strong indication of overall sample degradation, in which case the sample should fail.

Species (mass ratio)	rRNAs	Length [in kb]
Human (2.6)	18S/28S	1.9 / 5.0
Mouse (2.5)	18S/28S	1.9 / 4.7
Teleost fish (Zebrafish, *Danio rerio)* (2.3)	18S/28S	1.9 /4.1
Drosophila melanogaster	18S/28S	2.0 / 4.1*
Plant		
cytoplasmic (1.9)	18S/25S	1.9 / 3.7
chloroplasts ** (1.8)	16S/23S	1.5 / 2.7
Caenorhabditis elegans (2.0)	18S/26S	1.75 / 3.5
Yeast *(Saccharomyces cerevisiae)* (1.9)	18S/26S	2.0 /3.8
Bacteria *(E.coli) (1.9)*	16S/23S	1.5 /2.9

Table 1
Length variations of rRNAs from different species and tissue types.
Species names are followed by rRNA ratios (in brackets). Please note: * Drosophila 28S rRNA is split in 2 fragments, comigrating with 18S rRNA. ** Prominent bands only in RNAs from green tissues with high chloroplast content.

http://cp.chem.agilent.com/Library/applications/5989-1086EN.pdf

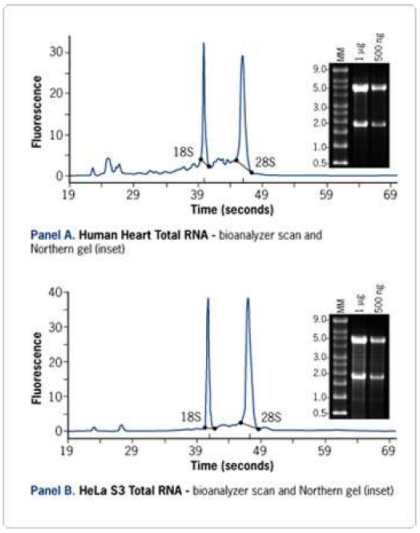


Figure 1. RNA Expression Profiles from Different Tissues. Denaturing agarose gel (inset) and Agilent bioanalyzer scan of Human Heart Total RNA (100 ng) (A) and HeLa cell line total RNA (B) isolated by multistep phenol extraction and glass fiber filter binding, respectively. The heart sample had a 28S:18S rRNA ratio of 1.51, and the HeLa cell sample had a 28S:18S rRNA ratio of 1.72.

http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rna-isolation/tech-notes/assessing-rna-quality.html

c. Note that there may be additional ribosomal peaks, especially if the RNA sample is derived from plant material which contains chloroplast ribosomal RNA:

Ribosomal RNA	Types of RNA
Cytosolic ribosomes	
Larger subunit	25S, 8S, 5S
Smaller subunit	18S
Chloroplasts ribosomes	
Larger subunit	23S, 5S
Smaller subunit	16S
Mitochondrial ribosomes	
Larger subunit	24S, 5S
Smaller subunit	18S, 5S

Table 1
Plant ribosomal RNA.

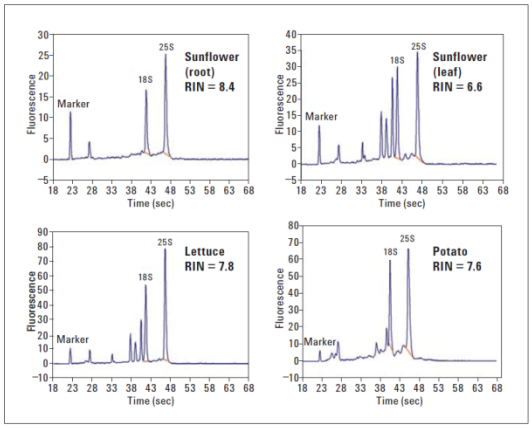


Figure 1
Electrophoretic separation of different plant total RNA using the RNA 6000 Nano Kit with the Plant RNA assay. Representative electropherograms indicate the position of 18S and 25S rRNA peaks. Unlabeled peaks correspond to additional ribosomal RNA. RIN values are shown.

http://www.chem.agilent.com/Library/applications/5990-8850EN.pdf

d. Fail if there is significant RNA degradation in the electropherogram (e.g., if there is an elevated threshold baseline). Refer to the RNA Integrity Number (RIN) value to determine if degradation is significant. The sample below with a RIN of 6 should fail due to both a poor rRNA ratio and an elevated baseline indicating significant sample degradation, as discussed previously. The RIN value should only be used as a reference however (do not fail a sample just because the RIN is 6 or below).

Please review the following document regarding RIN values: http://www.chem.agilent.com/en-us/Search/Library/_layouts/Agilent/PrimaryDocumentViewer.ashx?whid=37507

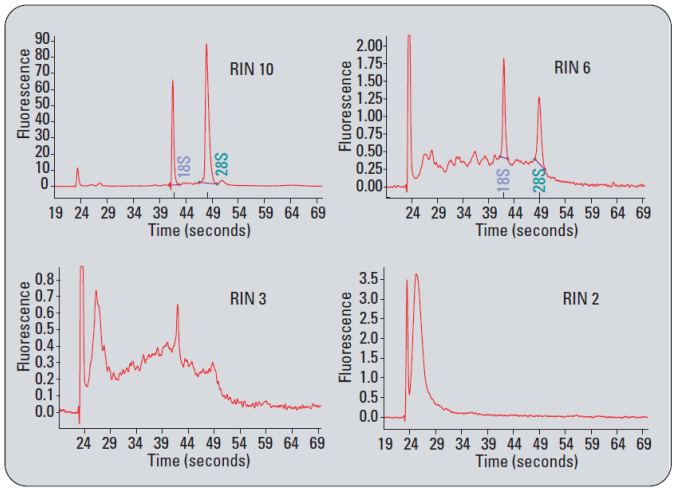
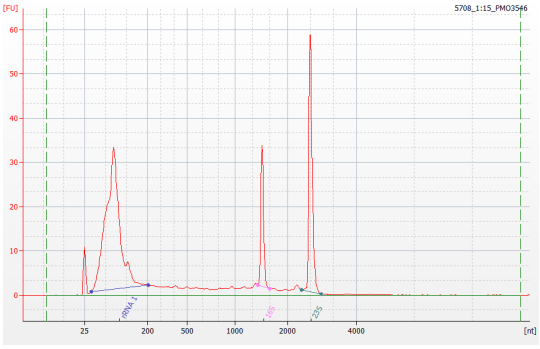


Figure 2
Sample electropherograms used to train the RNA Integrity Number (RIN) software. Samples range from intact (RIN 10), to degraded (RIN 2).

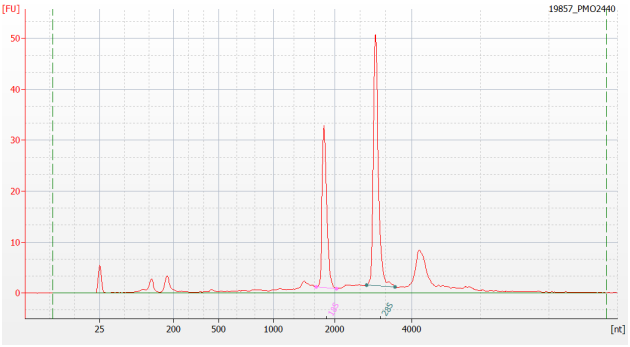
http://www.chem.agilent.com/en-us/Search/Library/_layouts/Agilent/PrimaryDocumentViewer.ashx?whid=37507

e. If there is a peak in the 5S region, it should not be larger than the 2 main rRNA peaks. For example, the following RNA sample, while borderline, would pass because the 5S region peak is not taller than the 16S or 23S peaks. However, if the 5S peak is any larger than this, or if there is an elevated baseline associated with the 5S region peak, which is a strong indicator of degradation, the sample should fail.



f. Fail if any genomic DNA (gDNA) contamination is present (peaks are typically larger than 4000 nt as in the electropherogram below). To confirm that a suspected gDNA peak is gDNA, perform DNase treatment and purification on a small RNA aliquot, and repeat the RNA Nano Chip run (please see Appendix for protocol for DNase treatment). Samples with gDNA contamination should be failed and can be returned to the collaborator for DNase treatment. The project manager should be

notified if there is gDNA contamination so they can consult with the collaborator on how their sample sets are treated.



5.15 For all Metatranscriptome RNA samples use the following specifications to determine if RNA samples pass or fail:

a. Fail if total RNA mass is insufficient for required aliquot size.
   i. 2µg ±10% aliquot mass for standard RNA-Seq with rRNA Depletion library construction.
   ii. 50ng ±10% aliquot mass for RNA-Seq without rRNA Depletion library construction.
   iii. 10ng ±10% aliquot mass for Low Input RNA-Seq library construction.
   iv. Note, thus far there have been no requests for Small RNA library construction from Metatranscriptome samples.
b. Fail if any gDNA contamination (please see above for details).

Appendix

DNase treatment of an aliquot of RNA that is suspected to contain genomic DNA contamination.

Materials & Reagents

Materials/Reagents/Equipment	**Supplier/Vendor**	**Stock Number**
RNase-Free DNase Set (DNase I, Buffer RDD)	Qiagen	79254
RNase-free Needle and Syringe (1 ml)	VWR	BD309623
RNeasy MinElute Cleanup Kit	Qiagen	74204
Ethanol	Sigma Aldrich	E7023-6X500ML

i. For suspected genomic DNA contamination in RNA sample, perform DNase treatment

a. Perform DNase treatment only on a small aliquot of the RNA sample (do not treat the entire sample). Create the smallest aliquot necessary so that the final concentration of RNA after the treatment will be enough to see on the Bioanalyzer Nano Chip.

b. This protocol follows the Qiagen RNeasy MinElute Cleanup Handbook instructions on page 24 and page 10 (http://www.qiagen.com/Products/Catalog/Sample-Technologies/RNA-Sample-Technologies/RNA-Cleanup/RNeasy-MinElute-Cleanup-Kit#resources). Review this protocol before proceeding.

c. Prepare DNase I stock solution and create aliquots.

   i. Do not open the DNase I glass vial. Using a needle and syringe, dissolve the lyophilized DNase I with 550µl of nuclease-free water (be very careful with the needle, it is very sharp!).

   ii. Do not vortex the DNase I. Mix gently by inverting the vial until all lyophilized DNase I is dissolved.

   iii. Remove the stock solution from the vial (can use syringe, or can open vial and remove with pipette). Create aliquots of 20µl each. Aliquots should be stored at -20^{0}C, and are for single use only (do not refreeze the aliquots after thawing).

d. Perform DNase treatment.

   i. Mix the following in a microcentrifuge tube:

      1. 87.5µl RNA
      2. 10µl Buffer RDD
      3. 2.5µl DNase I

   ii. Incubate at room temperature for 10 minutes.

e. Clean up the RNA according to protocol on page 10 of Qiagen RNeasy MinElute Cleanup Handbook, "RNA Cleanup and Concentration".

   i. Prepare Buffer RPE by adding 4 volumes of ethanol.

   ii. Add 350µl Buffer RLT to 100µl RNA sample treated with DNase. Mix well.

   iii. Add 250µl ethanol to the diluted RNA, and mix by pipetting. Proceed immediately to next step.

   iv. Transfer the sample to an RNeasy MinElute spin column. Close the lid, centrifuge for 15 seconds at > 10,000 rpm, and discard the collection tube and flow-through. Transfer the spin column (containing RNA) to a new 2ml collection tube.

   v. Add 500µl Buffer RPE to the spin column. Close the lid, and centrifuge for 15 seconds at > 10,000 rpm. Discard the flow-through.

   vi. Add 500µl ethanol to the spin column. Close the lid, and centrifuge for 2 minutes at > 10,000 rpm. Discard the collection tube and flow-through.

   vii. Place the spin column in a new 2ml collection tube. Open the lid, and centrifuge at full speed for 5 minutes. Discard the flow-through and collection tube.

viii. Place the spin column in a new 1.5ml collection tube. Add 14µl nuclease-free water to the center of the spin column. Close the lid, and incubate 1 minute. Centrifuge for 1 minute at > 10,000 rpm to elute the RNA.

f. Run the RNA sample on a Nano Chip as describe previously.

g. Confirm if the sample contained genomic DNA by comparing the electropherograms of before and after treatment: